JOURNAL OF VIROLOGY, Aug. 2011, p. 7942–7947 Vol. 85, No. 15
0022-538X/11/$12.00 doi:10.1128/JVI.02535-10

Genus-Specific Substitution Rate Variability among Picornaviruses▽†

Allison L. Hicks and Siobain Duffy*

Department of Ecology, Evolution, and Natural Resources, School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, New Jersey

Received 5 December 2010/Accepted 14 May 2011

Picornaviruses have some of the highest nucleotide substitution rates among viruses, but there have been no comparisons of evolutionary rates within this broad family. We combined our own Bayesian coalescent analyses of VP1 regions from four picornaviruses with 22 published VP1 rates to produce the first within-family meta-analysis of viral evolutionary rates. Similarly, we compared our rate estimates for the RNA polymerase 3D^{pol} gene from five viruses to four published 3D^{pol} rates. Both a structural and a nonstructural gene show that enteroviruses are evolving, on average, a half order of magnitude faster than members of other genera within the *Picornaviridae* family.

Members of the *Picornaviridae* family are the most common cause of human viral infections in developed countries (28, 39). Human picornaviruses produce symptoms ranging from mild respiratory illness to hemorrhagic conjunctivitis, myocarditis, acute flaccid paralysis, and neonatal organ failure (19, 27, 28, 33, 40, 41). Veterinary picornaviruses, such as foot-and-mouth disease virus (FMDV), encephalomyocarditis virus (EMCV), and porcine teschovirus (PTV), can have devastating effects on livestock (5, 8, 21).

Although picornaviruses such as poliovirus (PV) are known to evolve more rapidly than other viruses with single-stranded RNA (ssRNA) genomes (9, 29), little research has been conducted to investigate how they evolve more rapidly than other viruses with similarly error-prone RNA-dependent RNA polymerases (29, 57) or if certain picornaviruses evolve more rapidly than others. Understanding the evolutionary potentials and constraints of these important pathogens is imperative for the development of durable vaccines and effective treatment plans for individual pathogens (42). As even small, 3-fold differences in RNA virus mutation rates can have dramatic consequences, such as driving a population into lethal mutagenesis (7), similar differences in long-term evolutionary rates could indicate significantly dissimilar evolutionary potentials.

While viral evolutionary rates were previously calculated only by linear regression, modern simulation software such as BEAST (11) allows for the estimation of more complex models of viral evolution. These Bayesian coalescent programs can produce both estimated mean rates of evolution and 95% credibility intervals (CIs) that provide a measure of the variability around mean rates. Instead of comparing single-point estimates, now nonoverlapping CIs provide the strongest evidence that genes or organisms are evolving at different rates (11). Many substitution rate estimates have been published for picornaviruses, especially for the antigenically significant VP1 gene, which encodes the most external of the picornavirus structural proteins and interacts with cellular receptors (37, 49). Based on sequence availability in GenBank, four novel analyses were conducted, measuring the rate of evolution of the VP1 gene for two enteroviruses and producing the first rate estimates for the type species of the genera *Cardiovirus* and *Teschovirus*. Fewer previous analyses and more limited GenBank data were available for other genes. We conducted five novel analyses of the rate of evolution of the 3D^{pol} polymerase gene for two enteroviruses and the type species of *Aphthovirus*, *Hepatovirus*, and *Parechovirus*.

Partial VP1 gene sequences of two human enterovirus B serotypes (coxsackievirus B2 [CVB2] and CVB4), encephalomyocarditis virus, and porcine teschovirus, of the genera *Enterovirus*, *Cardiovirus*, and *Teschovirus*, respectively, were obtained from GenBank (Table 1; Fig. 1). Partial 3D^{pol} gene sequences of a human enterovirus A serotype (enterovirus 71 [EV71]), a human enterovirus C serotype (PV type 1 [PV1]), a foot-and-mouth disease serotype (FMDV-A), hepatitis A virus (HAV), and human parechovirus (HPeV), of the genera *Enterovirus*, *Aphthovirus*, *Hepatovirus*, and *Parechovirus*, respectively, were obtained from GenBank (Table 1; Fig. 1). Dates of isolation (years) were obtained from GenBank or from the paper that described the virus's isolation (2, 8, 13, 16–18, 22, 26, 31, 32, 35, 38, 45, 49, 52, 59, 61–63, 66, 68, 69). Dates are given in the taxon labels in supplementary figures S1 to S9. We excluded sequences from viruses that had been extensively passaged in the lab prior to sequencing. For each virus, partial gene sequences were manually aligned using Se-Al version 2.0a11 (A. Rambaut, Institute of Evolutionary Biology, University of Edinburgh, United Kingdom; http://tree.bio.ed.ac.uk/). Each alignment included VP1 or 3D^{pol} sequences from as many dated isolates as possible and was trimmed to preserve the reading frame. No recombination was detected by RDP 3.44 (43).

Modeltest version 3.7 (55) determined the best-fitting nucleotide substitution model for each alignment (by Akaike's information criterion). Estimated nucleotide substitution rates and maximum clade credibility (MCC) trees for each alignment were obtained using BEAST (11). Three demographic models (constant population size, exponential growth, and Bayesian skyline) and both strict and uncorrelated lognormal

* Corresponding author. Mailing address: 14 College Farm Rd., New Brunswick, NJ 08901. Phone: (732) 932-8165, ext. 318. Fax: (732) 932-0312. E-mail: duffy@aesop.rutgers.edu.

† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 25 May 2011.

TABLE 1. Alignments and results of substitution rate analyses of picornaviruses

Gene	Viral species[a]	No. of taxa	No. of nt[b]	Date range	Substitution model	Clock model	TMRCA (ybp)[c]	Substitution rate ($\times 10^{-3}$)[c]	*dN*/*dS*
VP1	Human enterovirus B (CVB2)	51	231	1946–2008	GTR+I+Γ	Relaxed	72 (60–100)	5.27 (3.57–7.06)	0.04
	Human enterovirus B (CVB4)	110	300	1959–2007	TrN+I+Γ	Relaxed	75 (60–95)	5.73 (4.18–7.29)	0.03
	Encephalomyocarditis virus	27	210	1986–2008	K80+I	Strict	247 (98–824)	1.61 (0.56–2.78)	0.03
	Porcine teschovirus	46	702	1957–2007	GTR+I+Γ	Strict	507 (205–832)	1.62 (0.63–2.75)	0.10
$3D^{pol}$	Human enterovirus A (EV71)	153	999	1986–2010	TN93+I+Γ	Relaxed	120 (97–139)	5.53 (4.29–6.67)	0.05
	Human enterovirus C (PV1)	51	534	1982–2006	GTR+I+Γ	Relaxed	45 (31–69)	11.68 (8.12–14.53)	0.03
	Foot-and-mouth disease virus (FMDV-A)	24	642	1971–2009	GTR+I+Γ	Strict	152 (33–424)	1.45 (0.70–2.24)	0.05
	Hepatitis A virus	25	402	1976–2008	TN93+I+Γ	Strict	746 (429–995)	0.89 (0.46–1.31)	0.03
	Human parechovirus	142	657	1975–2009	GTR+I+Γ	Relaxed	124 (88–212)	2.96 (1.88–3.92)	0.04

[a] Abbreviations are given for enterovirus and aphthovirus serotypes.
[b] nt, nucleotides.
[c] Mean substitution rates are shown with lower and upper 95% credibility interval bounds in parentheses.

relaxed molecular clock models were used as priors in simulations. The marginal likelihoods of these six analyses were compared using Bayes factors in Tracer version 1.5 (http://tree.bio.ed.ac.uk/software/tracer/). To ensure accuracy, two independent 200-million-chain runs (of four Markov chain Monte Carlo [MCMC] chains each) were performed for each set of priors. An additional control simulation with an empty alignment and the best-fitting priors was performed to ensure that the priors alone were not determining the results. All of our controls indicated that our BEAST results were informative and reproducible. A comparison between the selected MCC trees and bootstrap-supported (1,000 replicates) maximum likelihood (ML) trees created with the respective best-fit models of nucleotide substitution using PAUP* version 4.0b8 (D. L. Swofford, Sinauer Associates, Sunderland, MA) showed largely consistent relationships among isolates (see Figures S1 to S9 in the supplemental material).

The relaxed molecular clock was the best-fitting prior for CVB2, CVB4, EV71, PV1, and HPeV (logBF $>$ 10 [BF is Bayes factor]), but the strict molecular clock was preferred for EMCV, PTV, FMDV-A, and HAV (logBF $>$ 1.8; Table 1). The constant demographic model was preferred for FMDV-A, HAV, and HPeV (logBF $>$ 2), the exponential model was the best fitting for PV1 and EV71 (logBF $>$ 2), and the Bayesian skyline model was the best fitting for the remaining viruses (logBF $>$ 2). The times to the most recent common ancestor (TMRCA) varied from very short time scales for the enterovirus serotypes (all of their 95% CI ranges coalesce within 140 years before the present [ybp]) to hundreds of years for the species-level analyses of EMCV, PTV, and HAV (Table 1). The faster-coalescing enterovirus genes have higher substitution rates than both VP1 and $3D^{pol}$ of the nonenteroviruses, with nonoverlapping credibility intervals (Table 1).

A literature review yielded 22 picornavirus partial and full VP1 substitution rates, summarized in Fig. 2. Published VP1 substitution rates for coxsackievirus B5 (CVB5), echovirus 9 (E9), echovirus 11 (E11), echovirus 30 (E30), HAV, and HPeV were obtained via BEAST analyses similar to those used in this

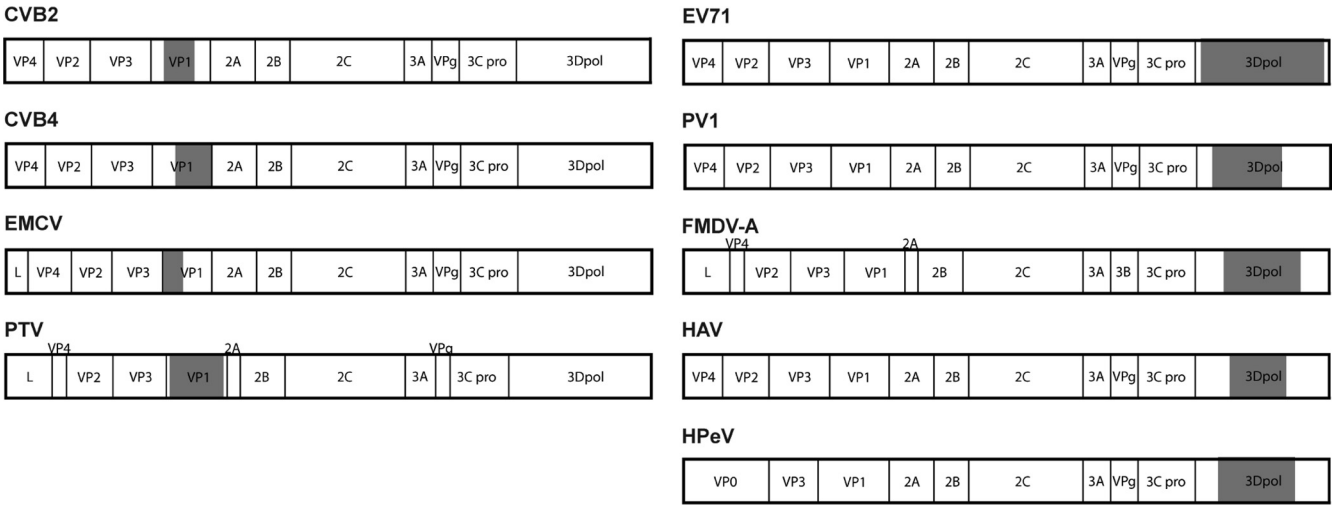

FIG. 1. Representations of the nine picornavirus genomes analyzed (~7 to 8 kb; ViralZone, Swiss Institute of Bioinformatics). Shading indicates portions of the VP1 or $3D^{pol}$ gene used in the study from the following viruses: coxsackieviruses B2 and B4 (CVB2 and CVB4), encephalomyocarditis virus (EMCV), porcine teschovirus (PTV), enterovirus 71 (EV71), poliovirus type 1 (PV1), foot-and-mouth disease virus type A (FMDV-A), hepatitis A virus (HAV), and human parechovirus (HPeV).

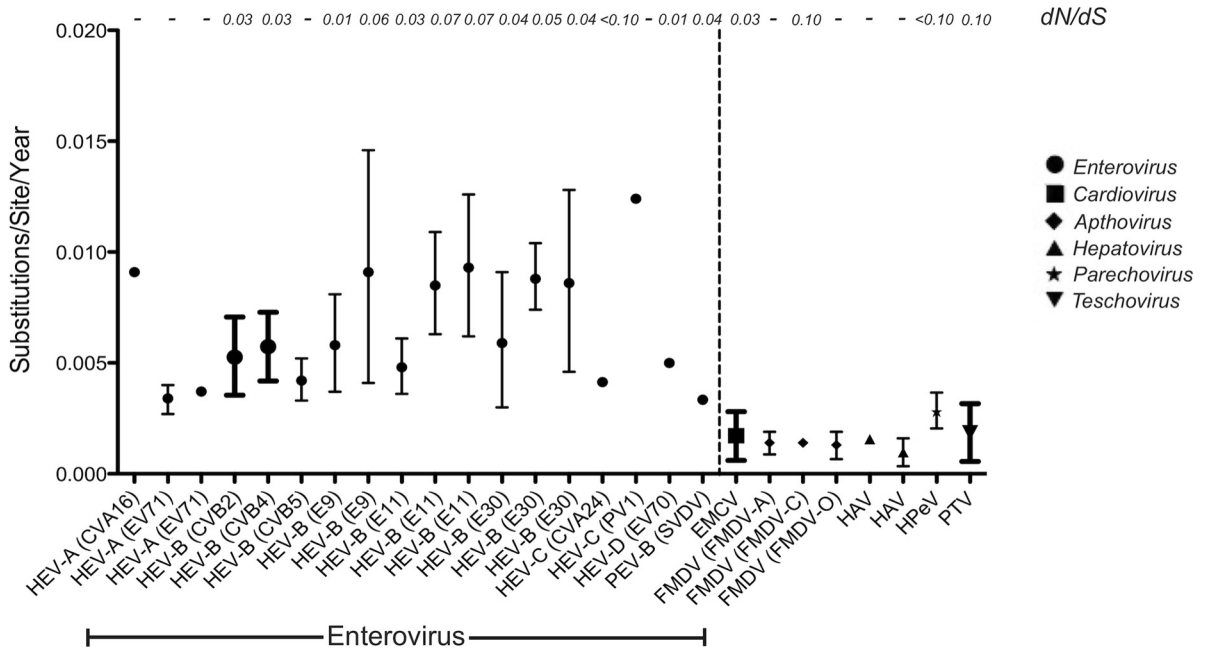

FIG. 2. Comparison of the VP1 nucleotide substitution rates of CVB2, CVB4, EMCV, and PTV (shown in bold) to published VP1 rates of other picornaviruses. Enteroviruses for which substitution rates are shown include serotypes coxsackievirus A16 (CVA16) (71), enterovirus 71 (EV71) (4, 29), coxsackievirus B5 (CVB5) (23), echovirus 9 (E9) (46), echovirus 11 (E11) (46), echovirus 30 (E30) (46, 47), coxsackievirus A24 (CVA24) (3), poliovirus type 1 (PV1) (50), enterovirus 70 (EV70) (64), and swine vesicular disease virus (SVDV) (70). Additionally, substitution rates for foot-and-mouth disease virus type A, type C, and type O (FMDV-A, FMDV-C, and FMDV-O) from the genus *Aphthovirus* (29, 44), hepatitis A virus (HAV) from the genus *Hepatovirus* (36, 48), and human parechovirus (HPeV) from the genus *Parechovirus* (15) are shown. Ninety-five percent confidence (EV71 and FMDV; calculated with TipDate) or credibility (all others; calculated with BEAST) intervals are shown where available. *dN*/*dS* ratios for the alignments that produced these rate estimates are shown in italics above, where available. Dashed line delineates the enteroviruses from other genera.

study (15, 23, 34, 46–48); those for EV71, FMDV-A, and FMDV-O were obtained via analyses performed in TipDate (58), a precursor to BEAST (29); and the remaining rates were estimated via linear regression (3, 4, 44, 50, 66, 70, 71). These mean rates of enterovirus VP1 evolution range from 3.40×10^{-3} to 1.19×10^{-2} nucleotide substitutions per site per year (ns/s/y), and mean VP1 rates for nonenteroviruses range from 9.76×10^{-4} to 2.79×10^{-3} ns/s/y. The average of the 18 enterovirus mean rates was 6.50×10^{-3} ns/s/y (standard deviation [SD] = 2.61×10^{-3}), while the average of the eight nonenterovirus mean rates was four times lower at 1.60×10^{-3} ns/s/y (SD = 5.33×10^{-4}). The only overlap of substitution rates between enteroviruses and all other picornaviruses occurs as the upper boundary of the HPeV 95% CI overlaps with the estimates for several enteroviruses, coxsackievirus A16 (CVA16), CVB2, E30, EV71, and swine vesicular disease virus (SVDV).

Only four $3D^{pol}$ substitution rates have been published, all from BEAST analyses, and from three different human enterovirus B (HEV-B) serotypes (47). The mean rates of enterovirus $3D^{pol}$ evolution range from 5.53×10^{-3} ns/s/y to 1.17×10^{-2} ns/s/y, and mean rates for that of nonenteroviruses range from 8.89×10^{-4} ns/s/y to 2.96×10^{-3} ns/s/y. The average of the six enterovirus mean rates was 7.99×10^{-3} ns/s/y (SD = 2.71×10^{-3}), while the average of our three nonenterovirus mean rates was again more than four times lower at 1.77×10^{-3} ns/s/y (SD = 1.07×10^{-3}). HPeV is again the only source of overlap, as its 95% CI includes the lower CI boundary of enteroviruses E9 and E30.

Overall, the mean rates of genomic evolution of both human and veterinary enteroviruses are consistently higher than those of members of the two other human-infecting genera (*Hepatovirus* and *Parechovirus*) and three veterinary genera in the *Picornaviridae* (Fig. 2 and 3). These higher rates are evinced despite a wide range of mean rates; there was more variability among mean rates of enterovirus evolution than among those of isolates from five other genera (the standard deviation for enterovirus rates was four times [VP1] and two times [$3D^{pol}$] greater than for the other genera). Our novel and collected VP1 rates are similar to published rates of the entire P1 structural region (15, 29, 30), and our $3D^{pol}$ rates echo that of an adjacent nonstructural region of a veterinary enterovirus (29). The striking similarity between evolutionary rates of structural and nonstructural picornavirus genes has not previously been discussed in the literature, but for each virus the rates were remarkably consistent with the estimated rates for both regions. Despite undoubtedly different selection pressures and potentially divergent evolutionary histories due to recombination (60), the VP1 and $3D^{pol}$ genes of individual picornaviruses appear to share an evolutionary rate. As more picornavirus whole-genome sequences become available, it will be interesting to see if additional genes support these evolutionary patterns.

While previous investigations of RNA virus evolutionary-

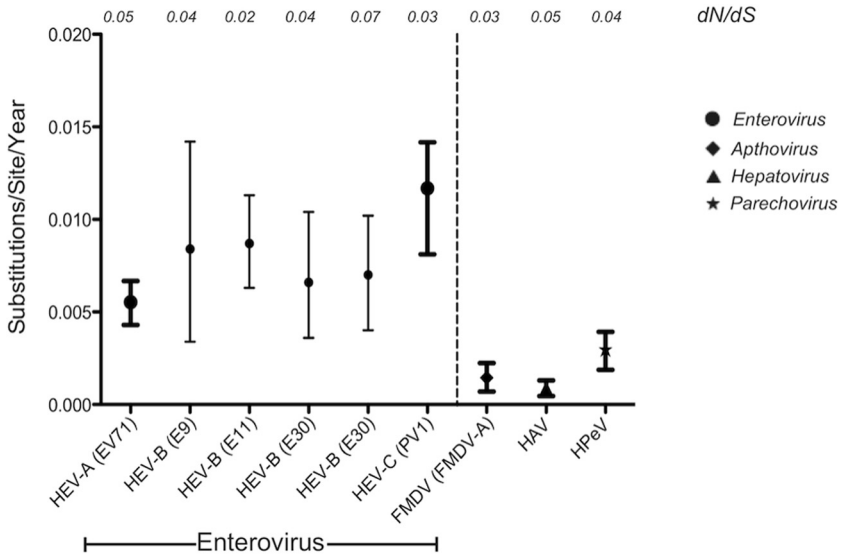

FIG. 3. Comparison of the $3D^{pol}$ nucleotide substitution rates of EV71, PV1, FMDV-A, HAV, and HPeV (shown in bold) to published $3D^{pol}$ rates of human enterovirus B serotypes echovirus 9 (E9), 11 (E11), and 30 (E30) (47). All are shown with 95% credibility intervals. *dN*/*dS* ratios are shown in italics above.

rate variability have been focused on the differences between viral families (29), our results demonstrate that significant long-term substitution rate variation can exist between related genera. The assumption that family members have identical rates underlies the recent estimations of long-term evolutionary rates of whole viral families, including those of the *Luteoviridae* (51), *Potyviridae* (20), and sobemoviruses (14). Our results discourage the continued presumption that evolutionary-rate differences among related genera or species must be negligible.

The immense serotype diversity of human pathogens within the genus *Enterovirus* (1) has created an imbalanced availability of sequence data within *Picornaviridae*. The genus *Enterovirus* is also by far the largest and most diverse genus in the family (2), which has allowed for more rate analyses of enteroviruses than of other genera. While all enterovirus substitution rates are of serotypes and most other available substitution rates are of species, the substitution rates of an enterovirus species should closely resemble the average of its serotype rates (30). However, our analyses showed that slower-evolving nonenterovirus species have much longer TMRCAs, which means they have had more time to become saturated at synonymous positions. Saturation reduces long-term substitution rate estimates and could explain some of the difference between fast-evolving enterovirus serotypes and the lower rates for species. Among the veterinary picornaviruses, three aphthovirus serotypes (FMDV-A, -C, and -O) still evolve significantly more slowly than the enterovirus serotype (SVDV). This serotype-to-serotype comparison confirms that the higher evolutionary rates of enteroviruses are not solely due to a difference in taxonomic scale (20).

Three common explanations for high per-year substitution rates are high per-generation mutation rates, high replication rates (increasing the number of generations per year), and positive selection (12). We tested whether our enterovirus alignments experienced positive selection, using the single likelihood, ancestor-counting, codon-based ML method on the Datamonkey web server (54). The estimated ratio of nonsynonymous to synonymous evolutionary changes (*dN*/*dS* ratio) for each of our alignments was very low ($\leq$0.1; Table 1), similar to published picornavirus ratios (Fig. 2 and 3), indicating strong purifying selection on both the VP1 and $3D^{pol}$ genes. No codons were found to be under positive selection in our nine analyses. Purifying selection is common in long-term RNA virus evolution: 73% of nucleotide substitutions from 46 RNA viruses were synonymous (29). While it is evident that negative selection on picornavirus genes does not preclude high nucleotide substitution rates, we have no evidence that positive selection on enteroviruses could explain their higher rates of evolution than those of other genera.

Due to their error-prone RNA-dependent RNA polymerases (RdRp), ssRNA viruses are known for high mutation rates, which are on the order of 10^{-5} to 10^{-3} mutations per nucleotide per replication event (10, 12, 25, 30, 57). In the absence of selection, the per-year substitution rate is solely a function of the per-replication mutation rate, so higher mutation rates can directly translate into higher substitution rates. It is possible that enteroviruses have evolved higher mutation rates than other picornaviruses. It has been suggested that the unusually low substitution rate of hepatitis A virus is due to its having evolved a significantly lower mutation rate than other picornaviruses (6). Indeed, the enterovirus poliovirus has a very high mutation rate that can be lowered through mutations in the RdRp (53, 67), indicating that its mutation rate is evolvable. On the other hand, atypical substitution rates within the family have also been attributed to tissue tropism (48). Tissue tropism can be easily linked to a virus's replication rate, as viruses that infect slowly dividing tissue will have a lower replication rate than viruses that infect rapidly dividing tissue (48). Because affinity for the gastrointestinal tract is a defining characteristic of enteroviruses, it is possible that higher enterovirus substitution rates are due to their primary tropism for intesti-

nal tissue, which has the highest turnover rate of all adult mammalian tissues (24, 65). This speculation is also supported by the fact that the fastest-evolving nonenterovirus, HPeV, frequently infects enteric tissue (15, 56) and was once classified as an enterovirus (15, 56). The mechanistic basis of the high enterovirus substitution rates remains an area of future research.

We thank Stefania Davia and an anonymous reviewer for their contributions to the EMCV analyses.

REFERENCES

1. **Blomqvist, S., C. Savolainen-Kopra, A. Paananen, T. Hovi, and M. Roivainen.** 2009. Molecular characterization of human rhinovirus field strains isolated during surveillance of enteroviruses. J. Gen. Virol. **90:**1371–1381.
2. **Bolanaki, E., C. Kottaridi, P. Markoulatos, L. Margaritis, and T. Katsorchis.** 2006. Evolution of 2B and 2C genomic parts of species B Coxsackie viruses. Phylogenetic study and comparison with other regions. Virus Genes **32:**249–259.
3. **Brandful, J. A., et al.** 1991. A study of the evolution of coxsackievirus A24 variant in Ghana by viral RNA fingerprinting analysis. Res. Virol. **142:**57–65.
4. **Brown, B. A., M. S. Oberste, J. P. Alexander, M. L. Kennett, and M. A. Pallansch.** 1999. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J. Virol. **73:**9969–9975.
5. **Chard, L. S., Y. Kaku, B. Jones, A. Nayak, and G. J. Belsham.** 2006. Functional analyses of RNA structures shared between the internal ribosome entry sites of hepatitis C virus and the picornavirus porcine teschovirus 1 Talfan. J. Virol. **80:**1271–1279.
6. **Cristina, J., and M. Costa-Mattioli.** 2007. Genetic variability and molecular evolution of hepatitis A virus. Virus Res. **127:**151–157.
7. **Cuevas, J. M., A. Moya, and R. Sanjuan.** 2009. A genetic background with low mutational robustness is associated with increased adaptability to a novel host in an RNA virus. J. Evol. Biol. **22:**2041–2048.
8. **Denis, P., et al.** 2006. Genetic variability of encephalomyocarditis virus (EMCV) isolates. Vet. Microbiol. **113:**1–12.
9. **Domingo, E.** 2007. Virus evolution, p. 389–421. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 5th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.
10. **Drake, J. W.** 1993. Rates of spontaneous mutation among RNA viruses. Proc. Natl. Acad. Sci. U. S. A. **90:**4171–4175.
11. **Drummond, A. J., and A. Rambaut.** 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. **7:**214.
12. **Duffy, S., L. A. Shackelton, and E. C. Holmes.** 2008. Rates of evolutionary change in viruses: patterns and determinants. Nat. Rev. Genet. **9:**267–276.
13. **Endo, K., et al.** 2007. Full-length sequences of subgenotype IIIA and IIIB hepatitis A virus isolates: characterization of genotype III HAV genomes. Virus Res. **126:**116–127.
14. **Fargette, D., et al.** 2008. Diversification of rice yellow mottle virus and related viruses spans the history of agriculture from the Neolithic to the present. PLoS Pathog. **4:**e1000125.
15. **Faria, N. R., M. de Vries, F. J. van Hemert, K. Benschop, and L. van der Hoek.** 2009. Rooting human parechovirus evolution in time. BMC Evol. Biol. **9:**164.
16. **Faustini, A., et al.** 2006. An outbreak of aseptic meningitis due to echovirus 30 associated with attending school and swimming in pools. Int. J. Infect. Dis. **10:**291–297.
17. **Fujiwara, K., et al.** 2001. Analysis of full-length hepatitis A virus genome in sera from patients with fulminant and self-limited acute type A hepatitis. J. Hepatol. **35:**112–119.
18. **García-Aguirre, L., and J. Cristina.** 2008. Analysis of the full-length genome of hepatitis A virus isolated in South America: heterogeneity and evolutionary constraints. Arch. Virol. **153:**1473–1478.
19. **Gavrilin, G. V., E. A. Cherkasova, G. Y. Lipskaya, O. M. Kew, and V. I. Agol.** 2000. Evolution of circulating wild poliovirus and of vaccine-derived poliovirus in an immunodeficient patient: a unifying model. J. Virol. **74:**7381–7390.
20. **Gibbs, A. J., D. Fargette, F. Garcia-Arenal, and M. J. Gibbs.** 2010. Time—the emerging dimension of plant virus studies. J. Gen. Virol. **91:**13–22.
21. **Goodwin, S., T. J. Tuthill, A. Arias, R. A. Killington, and D. J. Rowlands.** 2009. Foot-and-mouth disease virus assembly: processing of recombinant capsid precursor by exogenous protease induces self-assembly of pentamers in vitro in a myristoylation-dependent manner. J. Virol. **83:**11275–11282.
22. **Graff, J., A. Normann, S. M. Feinstone, and B. Flehmig.** 1994. Nucleotide sequence of wild-type hepatitis A virus GBM in comparison with two cell culture-adapted variants. J. Virol. **68:**548–554.
23. **Gullberg, M., et al.** 2010. Characterization of a putative ancestor of coxsackievirus B5. J. Virol. **84:**9695–9708.
24. **Heath, J. P.** 1996. Epithelial cell migration in the intestine. Cell Biol. Int. **20:**139–146.
25. **Holland, J., et al.** 1982. Rapid evolution of RNA genomes. Science **215:**1577–1585.
26. **Huang, S. C., et al.** 2008. Appearance of intratypic recombination of enterovirus 71 in Taiwan from 2002 to 2005. Virus Res. **131:**250–259.
27. **Iturriza-Gómara, M., B. Megson, and J. Gray.** 2006. Molecular detection and characterization of human enteroviruses directly from clinical samples using RT-PCR and DNA sequencing. J. Med. Virol. **78:**243–253.
28. **Jacques, J., et al.** 2008. Epidemiological, molecular, and clinical features of enterovirus respiratory infections in French children between 1999 and 2005. J. Clin. Microbiol. **46:**206–213.
29. **Jenkins, G. M., A. Rambaut, O. G. Pybus, and E. C. Holmes.** 2002. Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. J. Mol. Evol. **54:**156–165.
30. **Jorba, J., R. Campagnoli, L. De, and O. Kew.** 2008. Calibration of multiple poliovirus molecular clocks covering an extended evolutionary range. J. Virol. **82:**4429–4440.
31. **Joshi, M. S., A. M. Walimbe, and S. D. Chitambar.** 2008. Evaluation of genomic regions of hepatitis A virus for phylogenetic analysis: suitability of the 2C region for genotyping. J. Virol. Methods **153:**36–42.
32. **Karakasiliotis, I., E. Paximadi, and P. Markoulatos.** 2005. Evolution of a rare vaccine-derived multirecombinant poliovirus. J. Gen. Virol. **86:**3137–3142.
33. **Kew, O., et al.** 2002. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Science **296:**356–359.
34. **Kew, O. M., et al.** 1998. Prolonged replication of a type 1 vaccine-derived poliovirus in an immunodeficient patient. J. Clin. Microbiol. **36:**2893–2899.
35. **Knowles, N. J., et al.** 1998. Molecular analysis of encephalomyocarditis viruses isolated from pigs and rodents in Italy. Virus Res. **57:**53–62.
36. **Kulkarni, M. A., A. M. Walimbe, S. Cherian, and V. A. Arankalle.** 2009. Full length genomes of genotype IIIA hepatitis A virus strains (1995–2008) from India and estimates of the evolutionary rates and ages. Infect. Genet. Evol. **9:**1287–1294.
37. **Laine, P., S. Blomqvist, C. Savolainen, K. Andries, and T. Hovi.** 2006. Alignment of capsid protein VP1 sequences of all human rhinovirus prototype strains: conserved motifs and functional domains. J. Gen. Virol. **87:**129–138.
38. **LaRue, R., et al.** 2003. A wild-type porcine encephalomyocarditis virus containing a short poly(C) tract is pathogenic to mice, pigs, and cynomolgus macaques. J. Virol. **77:**9136–9146.
39. **Lewis-Rogers, N., and K. A. Crandall.** 2010. Evolution of Picornaviridae: an examination of phylogenetic relationships and cophylogeny. Mol. Phylogenet. Evol. **54:**995–1005.
40. **Lindberg, A. M., P. Andersson, C. Savolainen, M. N. Mulders, and T. Hovi.** 2003. Evolution of the genome of human enterovirus B: incongruence between phylogenies of the VP1 and 3CD regions indicates frequent recombination within the species. J. Gen. Virol. **84:**1223–1235.
41. **Lukashev, A. N., et al.** 2005. Recombination in circulating human enterovirus B: independent evolution of structural and non-structural genome regions. J. Gen. Virol. **86:**3281–3290.
42. **Mäkelä, P. H.** 2003. The molecular biologist against infectious disease. EMBO Rep. **4**(S1):S39–S42.
43. **Martin, D. P., et al.** 2010. RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics **26:**2462–2463.
44. **Martínez, M. A., et al.** 1992. Evolution of the capsid protein genes of foot-and-mouth disease virus: antigenic variation without accumulation of amino acid substitutions over six decades. J. Virol. **66:**3557–3565.
45. **Marturano, J., and L. Fiore.** 2002. Investigation of the presence of recombinant polioviruses in the hit population in Albania during the 1996 outbreak. J. Clin. Microbiol. **40:**316–317.
46. **McWilliam Leitch, E. C., et al.** 2009. Transmission networks and population turnover of echovirus 30. J. Virol. **83:**2109–2118.
47. **McWilliam Leitch, E. C., et al.** 2010. Evolutionary dynamics and temporal/geographical correlates of recombination in the human enterovirus echovirus types 9, 11, and 30. J. Virol. **84:**9292–9300.
48. **Moratorio, G., et al.** 2007. Bayesian coalescent inference of hepatitis A virus populations: evolutionary rates and patterns. J. Gen. Virol. **88:**3039–3042.
49. **Oberste, M. S., S. Penaranda, and M. A. Pallansch.** 2004. RNA recombination plays a major role in genomic change during circulation of coxsackie B viruses. J. Virol. **78:**2948–2955.
50. **Odoom, J. K., Z. Yunus, G. Dunn, P. D. Minor, and J. Martin.** 2008. Changes in population dynamics during long-term evolution of Sabin type 1 poliovirus in an immunodeficient patient. J. Virol. **82:**9179–9190.
51. **Pagán, I., and E. C. Holmes.** 2010. Long-term evolution of the *Luteoviridae*: time scale and mode of virus speciation. J. Virol. **84:**6177–6187.
52. **Peng, T., et al.** 2000. Characterization of enterovirus isolates from patients with heart muscle disease in a selenium-deficient area of China. J. Clin. Microbiol. **38:**3538–3543.
53. **Pfeiffer, J. K., and K. Kirkegaard.** 2003. A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleo-

tide analogs via increased fidelity. Proc. Natl. Acad. Sci. U. S. A. **100:**7289–7294.
54. **Pond, S. L., and S. D. Frost.** 2005. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics **21:**2531–2533.
55. **Posada, D., and K. A. Crandall.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817–818.
56. **Racaniello, V. R.** 2007. *Picornaviridae*: the viruses and their replication, p. 795–838. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 5th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.
57. **Sanjuán, R.** 2010. Mutational fitness effects in RNA and single-stranded DNA viruses: common patterns revealed by site-directed mutagenesis studies. Philos Trans. R. Soc. Lond. B Biol. Sci. **365:**1975–1982.
58. **Schneider, W. L., and M. J. Roossinck.** 2001. Genetic diversity in RNA virus quasispecies is controlled by host-virus interactions. J. Virol. **75:**6566–6571.
59. **Shimizu, H., et al.** 2004. Circulation of type 1 vaccine-derived poliovirus in the Philippines in 2001. J. Virol. **78:**13512–13521.
60. **Simmonds, P.** 2006. Recombination and selection in the evolution of picornaviruses and other mammalian positive-stranded RNA viruses. J. Virol. **80:**11124–11140.
61. **Singh, S., V. T. Chow, M. C. Phoon, K. P. Chan, and C. L. Poh.** 2002. Direct detection of enterovirus 71 (EV71) in clinical specimens from a hand, foot, and mouth disease outbreak in Singapore by reverse transcription-PCR with universal enterovirus and EV71-specific primers. J. Clin. Microbiol. **40:**2823–2827.
62. **Singh, S., C. L. Poh, and V. T. Chow.** 2002. Complete sequence analyses of enterovirus 71 strains from fatal and non-fatal cases of the hand, foot and mouth disease outbreak in Singapore (2000). Microbiol. Immunol. **46:**801–808.
63. **Stene-Johansen, K., T. O. Jonassen, and K. Skaug.** 2005. Characterization and genetic variability of hepatitis A virus genotype IIIA. J. Gen. Virol. **86:**2739–2745.
64. **Takeda, N., M. Tanimura, and K. Miyamura.** 1994. Molecular evolution of the major capsid protein VP1 of enterovirus 70. J. Virol. **68:**854–862.
65. **van der Flier, L. G., and H. Clevers.** 2009. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu. Rev. Physiol. **71:**241–260.
66. **van Rensburg, H., et al.** 2002. Genetic heterogeneity in the foot-and-mouth disease virus Leader and 3C proteinases. Gene **289:**19–29.
67. **Vignuzzi, M., J. K. Stone, and R. Andino.** 2005. Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications. Virus Res. **107:**173–181.
68. **Wu, Y., et al.** 2010. The largest outbreak of hand, foot and mouth disease in Singapore in 2008: the role of enterovirus 71 and coxsackievirus A strains. Int. J. Infect. Dis. **14:**e1076–e1081.
69. **Yan, J. J., J. R. Wang, C. C. Liu, H. B. Yang, and I. J. Su.** 2000. An outbreak of enterovirus 71 infection in Taiwan 1998: a comprehensive pathological, virological, and molecular study on a case of fulminant encephalitis. J. Clin. Virol. **17:**13–22.
70. **Zhang, G., D. T. Haydon, N. J. Knowles, and J. W. McCauley.** 1999. Molecular evolution of swine vesicular disease virus. J. Gen. Virol. **80:**639–651.
71. **Zhang, Y., et al.** 2010. Molecular evidence of persistent epidemic and evolution of subgenotype B1 coxsackievirus A16-associated hand, foot, and mouth disease in China. J. Clin. Microbiol. **48:**619–622.